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Award Number: W81XWH-11-1-0199

TITLE: Role of Protein Synthesis Initiation Factors in Dietary Soy Isoflavone-Mediated Effects on Breast Cancer Progression.

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REPORT DATE: August 2014

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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| REPORT DOCUMENTATION PAGE | | | <i>Form Approved</i> OMB No. 0704-0188 | |
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| 1. REPORT DATE August 2014 | | 2. REPORT TYPE Annual Summary | | 3. DATES COVERED 1 March 2011 – 31 May 2014 |
| 4. TITLE AND SUBTITLE Role of Protein Synthesis Initiation Factors in Dietary Soy Isoflavone-Mediated Effects on Breast Cancer Progression. | | | 5a. CONTRACT NUMBER | |
| | | | 5b. GRANT NUMBER W81XWH-11-1-0199 | |
| | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Columba de la Parra Simental Email: columba.delaparra@upr.edu | | | 5d. PROJECT NUMBER | |
| | | | 5e. TASK NUMBER | |
| | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Puerto Rico, San Juan San Juan, PR 00936-5067 | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | | |
| 13. SUPPLEMENTARY NOTES | | | | |
| 14. ABSTRACT The role of dietary soy in breast cancer has been the subject of intense investigation and is thought to be cancer preventive. However, the beneficial effects of soy on established breast cancer is controversial. The goal of this research is to investigate the potential molecular mechanisms by which soy isoflavones affect established breast cancers. A previous study by our laboratory reported that dietary genistein reduced tumor growth and metastasis and down regulated cancer promoting genes in a nude mouse model with tumors established from MDA-MB-435 metastatic cancer cells. In contrast, daidzein increased primary ammary tumor growth and metastasis, and significantly upregulated genes that regulate proliferation and protein synthesis including eukaryotic initiation factor eIF4F members. Our hypothesis is that soy isoflavones modulate breast cancer progression by specific regulation of the eIF4F eukaryotic initiation factor complex to affect the synthesis of cancer regulatory proteins. We have reported that equol, a metabolite of the soy isoflavone daidzein, may advance breast cancer progression via upregulation of the eukaryotic initiation factor eIF4G. In estrogen receptor (-) metastatic breast cancer cells, elevated eIF4G levels in response to equol were associated with an increase in cell viability and the translation of specific mRNAs with internal ribosome entry sites (IRES) including the transcription factor c-Myc, a central regulator of cancer malignancy. Herein, we show that eIF4G knockdown results in a marked reduction of the equol-mediated increase in protein synthesis initiation of specific IRES-dependent pro-cancer molecules, without affecting the increases in c-Myc levels and cell viability. Knockdown of c-Myc abrogated the increased cell viability in response to equol. Our findings implicate c-Myc and eIF4G in the cancer promoting effects of equol in metastatic breast cancer via regulation of protein synthesis initiation of molecules that control cancer progression. Training: Ph.D. Dissertation was written, edited, and successfully defended. | | | | |
| 15. SUBJECT TERMS- Key Words: breast cancer, equol, protein synthesis initiation, translation, eIF4G, c-Myc , cap-independent protein synthesis, | | | | |
| 16. SECURITY CLASSIFICATION OF: | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON USAMRMC |

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| a. REPORT U | b. ABSTRACT U | c. THIS PAGE U | UU | 38 | 19b. TELEPHONE NUMBER <i>(include area code)</i> |
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more ER β affinity than its precursor daidzein (15-17), also rodents are efficient producers of equol, but, not all humans have the gut microflora necessary to convert daidzein to equol, and 30–50% of humans are equol producers (18, 19). The soy isoflavone equol significantly increased eIF4G expression in MDA-MB-231 and MDA-MB-435 cells by ~1.8 and 1.3 fold respectively. The increase in eIF4G in the MDA-MB-231 cell line was more modest and not statistically significant but consistent at similar concentrations (>10 μ M). It is possible that the presence of ER in the MDA-MB-231 cell line may exert a differential effect on equol-mediated eIF4G expression. The protein levels of eIF4E and its inhibitory protein 4E-BP remained unchanged at all concentrations of equol tested, indicating a specific effect on eIF4G expression. Elevated eIF4F expression can impact cancer progression by increased protein synthesis even under the oxidative and nutrient stress conditions in solid tumors (18). Overexpression of eIF4GI is critical for the cap-independent mode of translation initiation in eukaryotic cells that bypasses the requirement for cap-dependent scanning and allows the 40S ribosome to be directly recruited via Internal Ribosome Entry Sites (IRESs). (9, 20-22). Interestingly, many mRNAs with IRESs code for several proteins related to cellular bioenergetics, survival, and proliferation such as, eIF4G, BCL2, Bcl-XI, C-Myc, p120 catenin, vascular endothelial growth factor (VEGF) etc (9, 23, 24). Even though we demonstrated that the mTOR signaling is not regulated by soy isoflavones, the levels of eIF4G are increased by the daidzein metabolite equol, therefore, we continued testing in our second and third year of the award the original hypothesis that soy isoflavones modulate breast cancer progression by specific regulation of the eIF4F eukaryotic initiation factor complex to affect the synthesis of cancer regulatory proteins (Fig. 2).

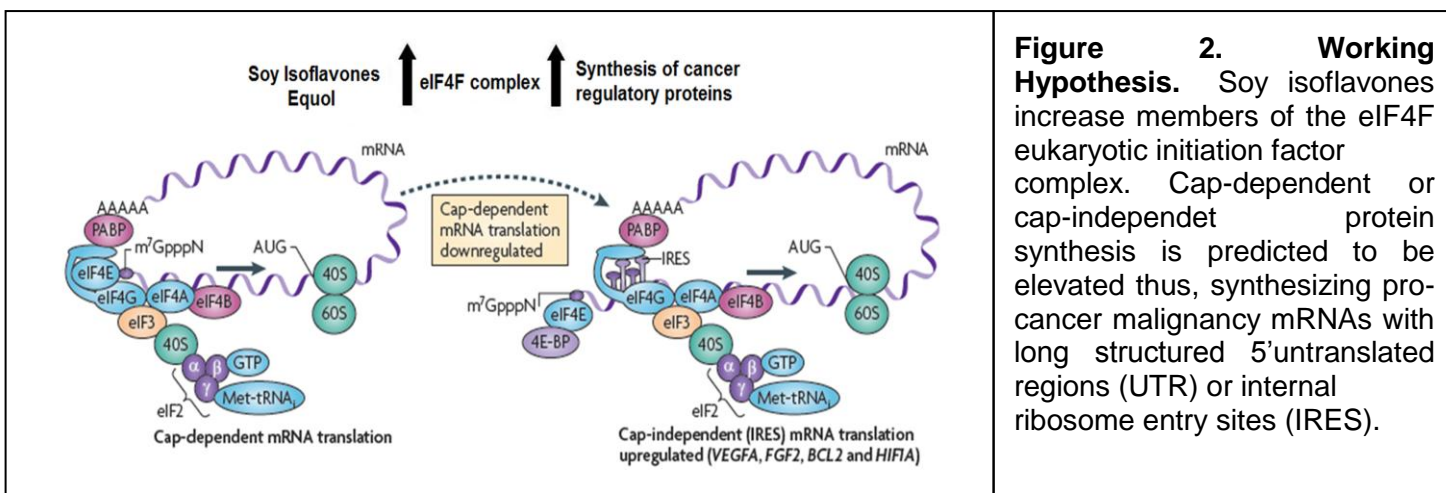
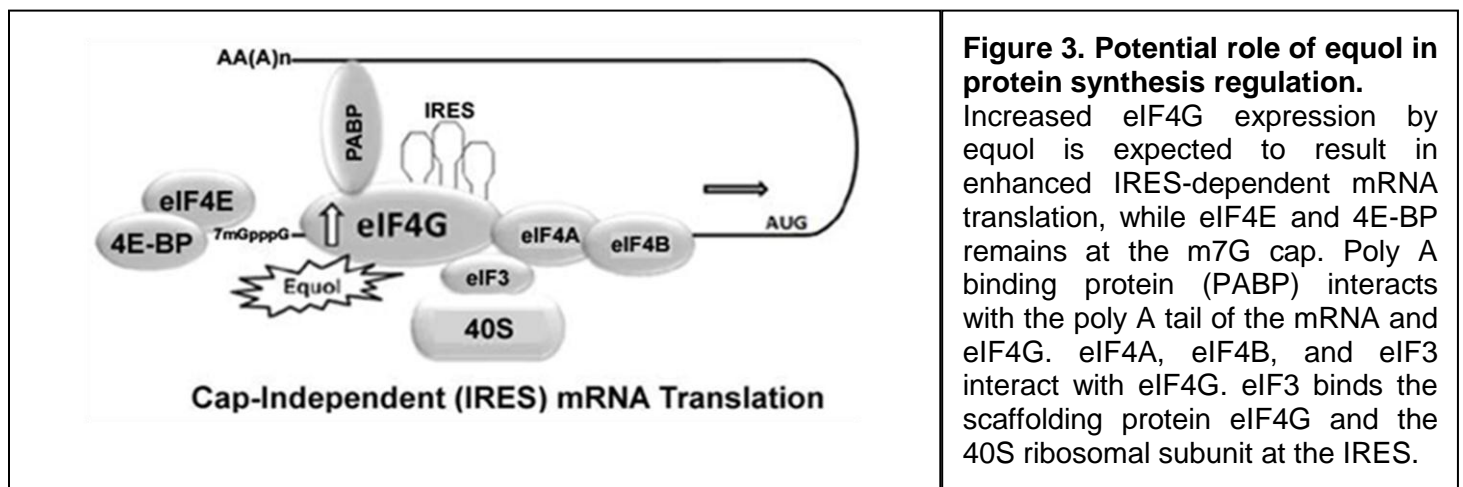


Figure 2. Working Hypothesis. Soy isoflavones increase members of the eIF4F eukaryotic initiation factor complex. Cap-dependent or cap-independent protein synthesis is predicted to be elevated thus, synthesizing pro-cancer malignancy mRNAs with long structured 5'untranslated regions (UTR) or internal ribosome entry sites (IRES).

Specific Aim 2: Investigate the effects of genistein, daidzein, glycitein or combined soy isoflavones on expression of mRNAs that are specifically regulated by the eIF4F initiation complex. The objective of this aim was to investigate whether soy isoflavones direct preferential translation of cancer promoting genes. **Completed.**

To investigate the effect of the overexpressed eIF4F complex on translation of mRNAs sensitive to elevated eIF4F initiation factors, as we described in the previous annual report, we investigated the expression of pro-cancer molecules with internal ribosomal entry sites (IRES) containing mRNAs. Since the daidzein metabolite equol had a drastic and significant effect on eIF4G expression, which is related to enhanced cap-independent translation (9, 21, 22), the effect of equol was analyzed on protein and mRNA levels of molecules that regulate cancer cell survival and proliferation in MDA-MB435 cells. Equol, upregulated protein expression of IRES-containing mRNAs: survivin, c-Myc, Bcl-2, Bcl-XL, Cyclin D, VEGF, and total and active p120 catenin, in the MDA-MB-435 cells by ~1.3-2.0-fold compared to vehicle. Of note was the dramatic increase in c-Myc protein expression by ~1.8-fold at all concentrations of equol tested (1-50 μ M). The expression of representative IRES positive mRNAs determined by qRT-PCR of cell lysates following vehicle or equol treatment demonstrated that IRES containing mRNAs CCND1 (Cyclin D), CTNND1 (p120 catenin), and VEGF did not change in response to equol. This suggested that the changes in protein levels of these molecules with mRNAs that have been shown to contain IRES sites are due to post-transcriptional regulation. However, 5-50 μ M equol upregulated gene expression of eIF4G and c-Myc, implicating equol in regulation of eIF4G and c-Myc at the transcription level.

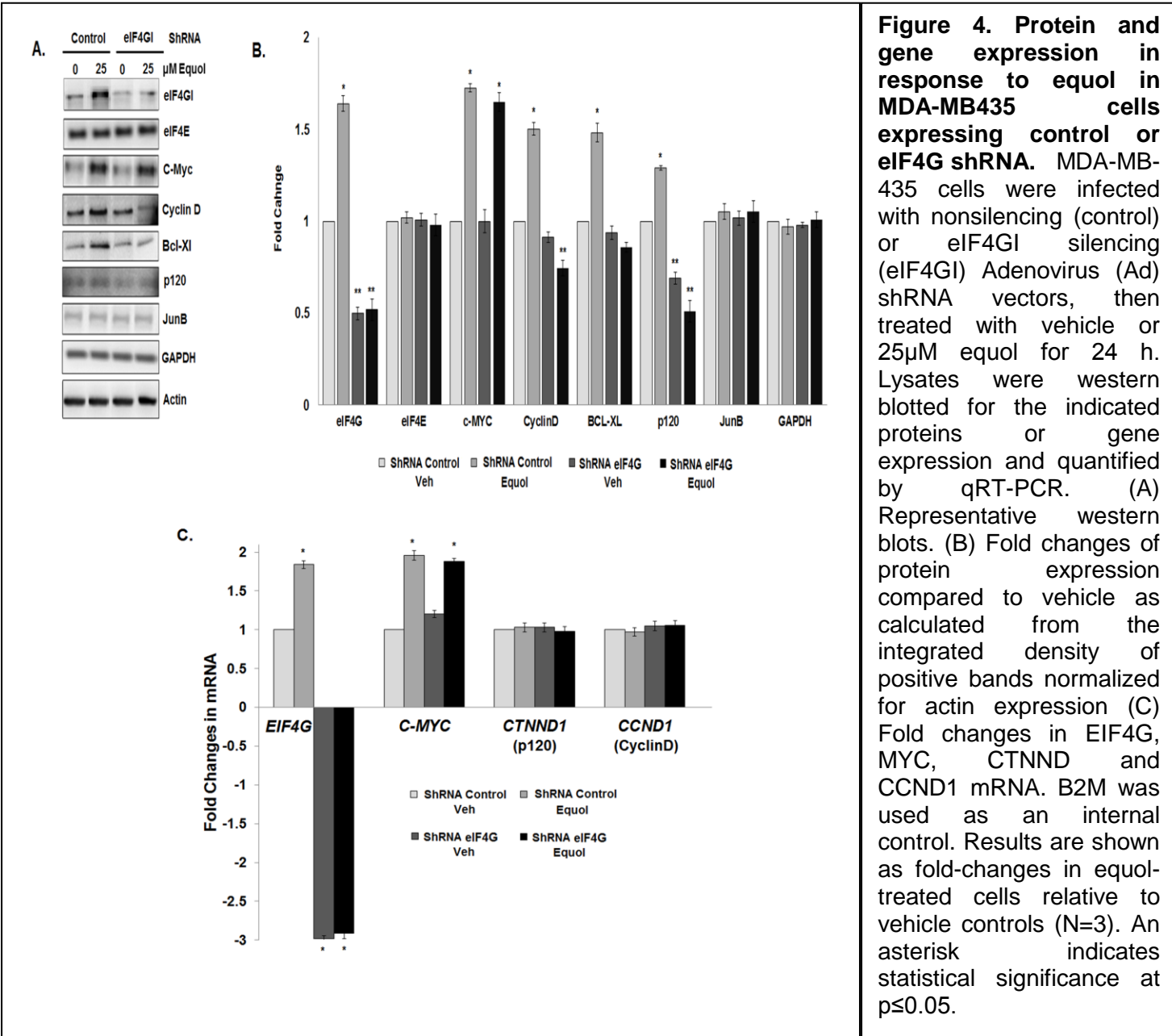
Polysomal distribution and expression of mRNAs known to be sensitive to eIF4F levels: The effect of equol on increased protein levels of IRES-containing mRNAs saturated at 25 μ M equol, therefore we selected these conditions for the subsequent *in vitro* assays. This concentration of equol has been found in the urine of humans after consumption of soy foods (25). To determine whether the up-regulated eIF4G levels in response to equol resulted in increased protein synthesis initiation, we performed polysomal fractionations of cell lysates after vehicle or equol. In the previous annual report, we showed that compared with vehicle treatments, equol increased the total mRNA associated with the polysomal fractions. qRT-PCR analyses for GAPDH, CTNND1, and EIF4G (B2M as the control) demonstrated that the IRES containing mRNAs CTNND1 and EIF4G, but not GAPDH, were associated with the heavier polysomal fractions from equol-treated cells by 1.5-fold and 1.7-fold, respectively. Similar to the results on protein expression, association of the IRES-negative GAPDH mRNA was not changed by equol treatment. Therefore, the observed equol-mediated up-regulation of p120-catenin protein expression without changes in CTNND1 gene expression may indicate preferential synthesis of IRES containing mRNAs in equol-treated cells. The enhanced affinity of EIF4G for the polysome fractions indicates that the EIF4G mRNA elevated in response to equol is translated into protein, thus accounting for the elevated eIF4G protein levels. Moreover, we investigated the possibility that equol-stimulated eIF4G expression may contribute to cap-independent translation. The synthetic *m7GTP* co-capture assays and the Dual luciferase assays for cap-dependent and cap-independent, IRES-mediated protein synthesis, described in our previous reports, demonstrated that the elevated eIF4G in response to equol was not associated with eIF4E or 4E-BP in 5' cap co-capture assays. The IRES-dependent protein synthesis was increased by equol in dual luciferase assays. Therefore, upregulation of eIF4G by equol may result in increased translation of pro-cancer mRNAs with IRESs (Fig.3). Nevertheless, this data does not rule out additional effects of equol on cap-dependent protein synthesis initiation, protein stability, or gene expression.



Specific Aim 3: Demonstrate that differential regulation of eIF4E and (or) eIF4G levels by soy isoflavones can directly contribute to breast cancer progression. The objective of this aim was to determine if *whether soy isoflavones modulate breast cancer progression by direct modulation of eIF4E and (or) eIF4G.* **Completed.**

As reported in the previously submitted and accepted annual report, we initiated this aim by investigating if the regulation of eIF4G that is elevated in response to equol, contribute to breast cancer progression. Using eIF4G1 shRNA vectors (kindly provided by Dr. Robert Schneider-New York University Langone Medical Center, NY) we were able to knockdown eIF4G1 protein expression by ~65%, compared to cells expressing vector controls (Fig. 4A).

The MDA-MB-435 cells were analyzed for protein expression levels of members of eIF4F complex and pro-cancer molecules from IRES containing mRNA that were previously shown to be upregulated by equol. eIF4G silencing caused a significantly reduction in expression IRES containing molecules: CyclinD, BCL-XI, -p120 in a statistically significant manner (Fig 4A,B). The protein expression of GAPDH, JunB and Actin from mRNAs with short 5'UTR lacking an IRES were not affected by equol treatment or eIF4G silencing. These results



suggest that elevated eIF4G levels are essential for the significant increase in protein expression of IRES-containing cancer promoting molecules, in response to equol treatment. Interesting, the equol-mediated ~1.7-fold increase in c-Myc, remained elevated regardless of the eIF4G knockdown and the eIF4E protein levels were not affected (Fig 4A). To determine whether silencing eIF4G may affect the molecules tested in Fig. 4A at the transcriptional level, we quantified the mRNA levels of eIF4G, C-MYC, CTNND1 (p120) and CCND1(CyclinD) from MDA-MB-435 cancer cells expressing control or eIF4G shRNA, treated with vehicle or 25μM equol. Figure 4C demonstrates that the ~1.8-fold increase of eIF4G by equol was abolished in cells expressing eIF4G shRNA, eIF4G expression was reduced by ~-3-fold in both vehicle and equol-treated cells. The mRNA levels of CTNND1 (p120) and CCND1 (CyclinD) were not affected by equol treatment or eIF4G knockdown, indicating that cyclin D and p120 expression was regulated at the translational level. However, mRNA levels of c-Myc increased by~1.9-fold in response to equol in both control and cells with eIF4G knockdowns, confirming that the equol-mediated upregulation in c-Myc protein levels is independent of eIF4G.

IRES dependent translation after eIF4G silencing- Dual Luciferase Assays:

In order to determine whether equol was regulated through the excess of eIF4G in the cytosol by cap-independent protein synthesis, we performed dual luciferase assays for cap-dependent and IRES-dependent

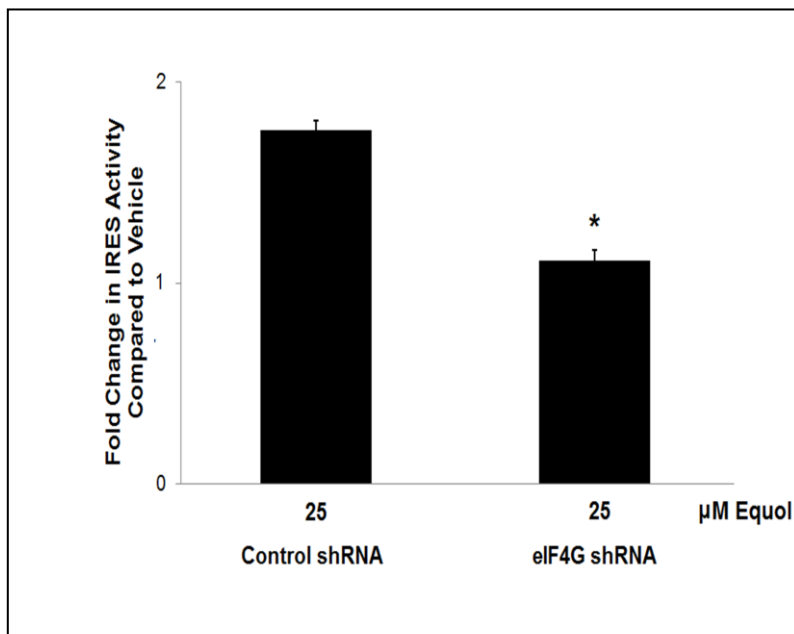


Figure 5. Effect of eIF4G knockdown on IRES-dependent protein synthesis in response to equol. MDA-MB-435 cells with control or eIF4G knockdowns expressing a plasmid with a cap-dependent Renilla luciferase (RLuc) followed by a 5'UTR containing the p120 catenin IRES driving a firefly luciferase (FLuc) or control plasmid without an IRES, were treated with vehicle or equol for 24 h. Cells were lysed, and the relative IRES activity analyzed as 570 nm FLuc/480 nm RLuc. IRES activity was quantified relative to control activity for vehicle or equol treated cells. Results show fold change in IRES activity compared to vehicle for N=3 \pm SEM. An asterisk indicates statistical significance of $p \leq 0.05$.

protein synthesis on MDA-MB-435 cells expressing control or eIF4G shRNA, treated with vehicle or equol. We finished and confirmed the preliminary data presented in our second annual report that equol increased IRES dependent translation in cells expressing control shRNA by ~1.7-fold, while this increase was significantly reduced when eIF4G was silenced (Fig.5). Thus, the elevated protein synthesis of mRNAs with IRESs can be attributed to the increased eIF4G in response to equol.

Polysome profiles for control and eIF4G-silenced MDA-MB-435 cells treated with vehicle or equol:

To investigate whether protein synthesis initiation might be sensitive to the reduction in eIF4G levels, we performed polysome profiles for control and eIF4G-silenced MDA-MB-435 cells treated with vehicle or equol. Fig. 6 showed that equol increased the total mRNA associated with the polysomal fractions compared with vehicle, especially in the cells expressing a control shRNA.

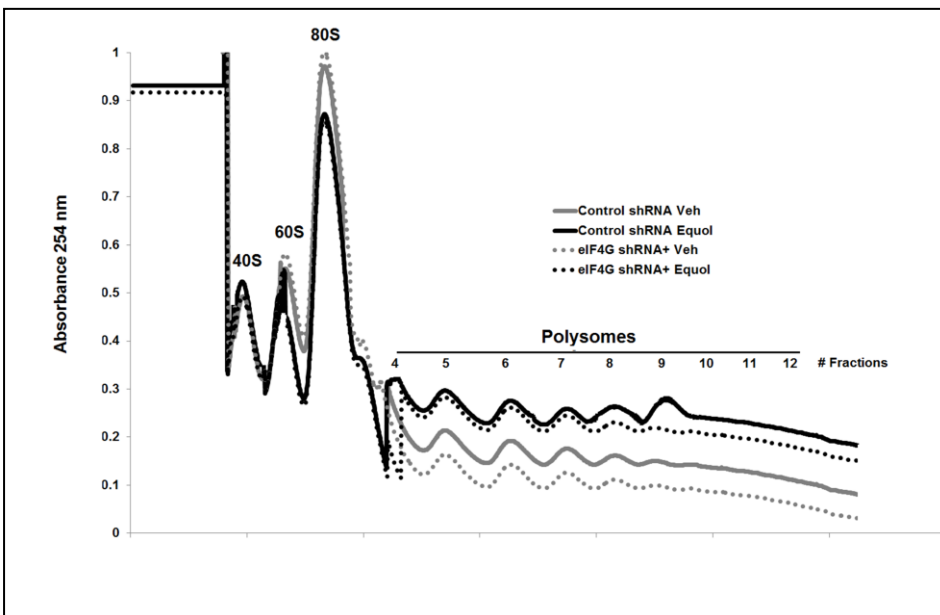
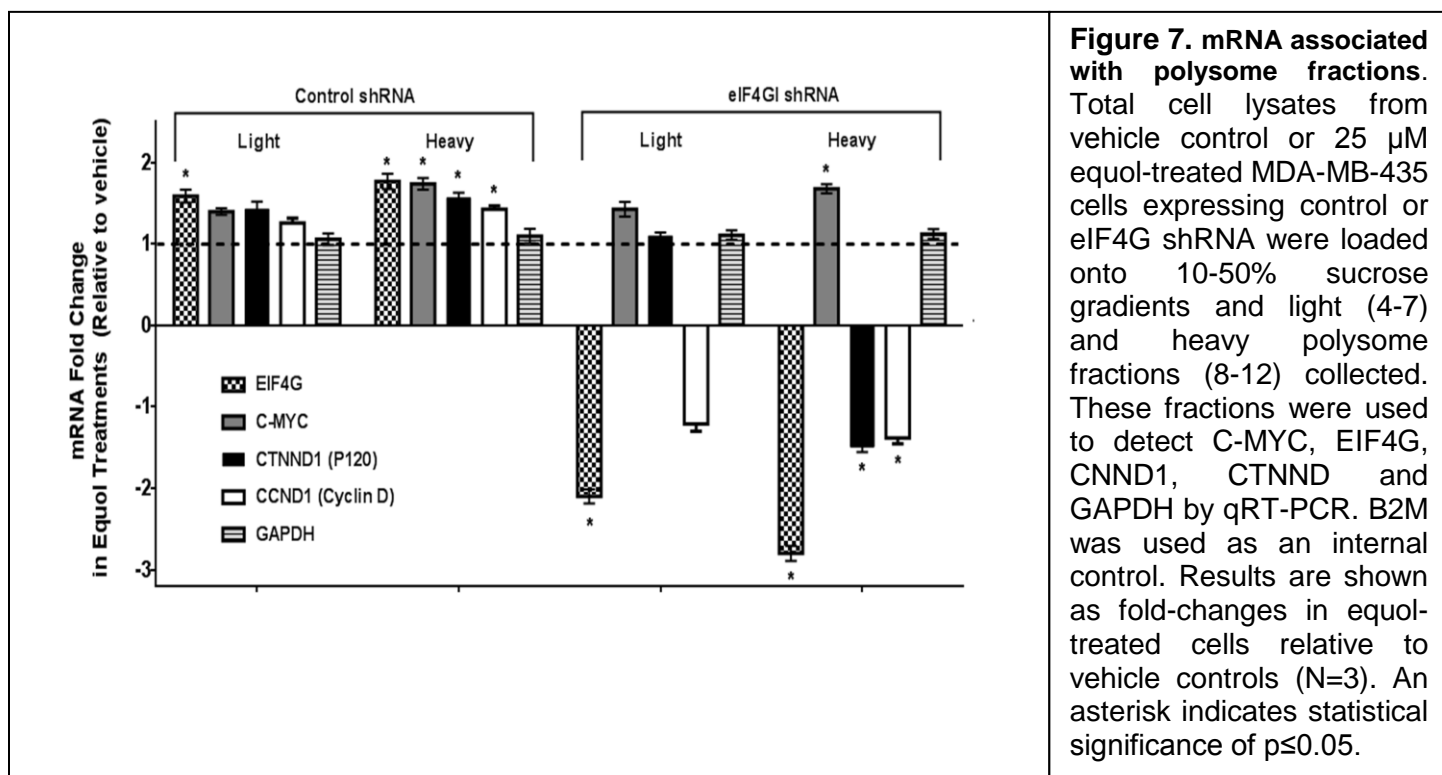


Figure 6. Analysis of polysome profiles in response to vehicle or equol in MDA-MB-435 cells expressing control or eIF4G shRNA. Total cell lysates from vehicle control or 25 μM equol-treated MDA-MB-435 cells expressing control or eIF4G shRNA were loaded onto 10-50% sucrose gradients and light (4-7) and heavy polysome fractions (8-12) collected. Results are shown as fold-changes in equol-treated cells relative to vehicle controls (N=3). An asterisk indicates statistical significance of $p \leq 0.05$.

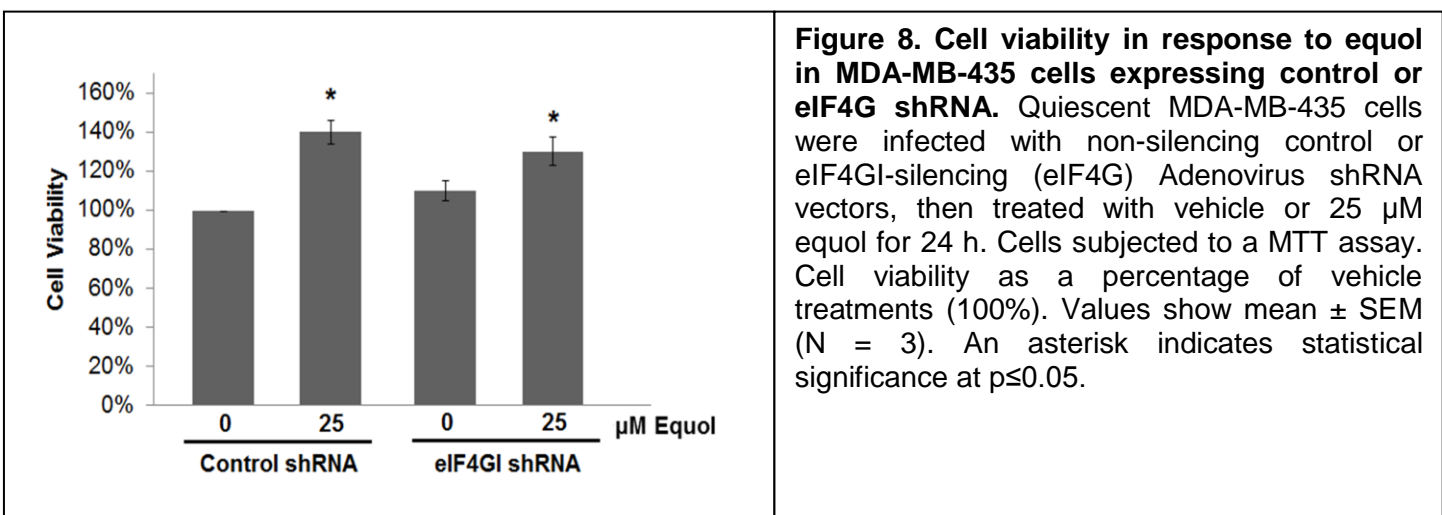
The total RNA associated with polysomes was slightly decreased in equol-treated MDA-MB-435 cells with eIF4G knockdown compared to cells expressing control shRNA. In the vehicle treated cells, eIF4G knockdown resulted in decreased association of RNA with both light and heavy polysomal fractions, compared to cells expressing a control shRNA. To identify specific mRNAs that were differentially associated with the polysome

fractions in control or eIF4G knockdown cells, real-time quantitative (q) RT-PCR was performed for the IRES

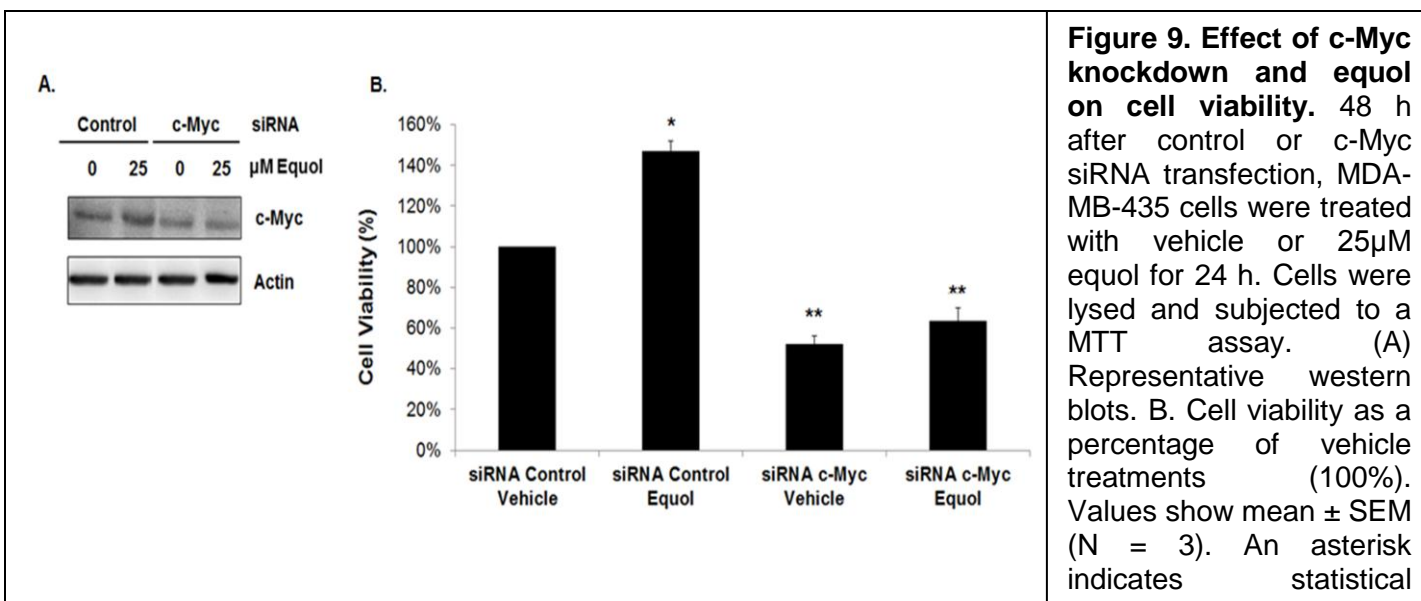


containing mRNAs: CTNND1, CCND1, EIF4G, C-MYC, and the non IRES containing housekeeping genes GAPDH and B2M. Figure 7 demonstrates that in the cells expressing control shRNA, equol treatment increased the association of eIF4G with both light and heavy polysomal fractions (~1.7-fold), while the association of c-Myc, CTNND1, and CCND1 with the heavy polysomal fraction was increased by ~1.5-1.8-fold. In cells expressing eIF4G shRNA, the reduction in eIF4G levels was reflected in decreased polysome association of CTNND1 and CCND1 by ~ -1.2 fold in the light fractions and by ~ -1.4 fold in the heavy fractions. However, the equol-stimulated c-Myc mRNA levels remained increased by 1.4 and 1.7 fold in the light and heavy fractions compared with vehicle controls, even in the cells with eIF4G knockdown. Association of the IRES-negative GAPDH mRNA was not changed by equol treatment. The observed decreased polysome association of mRNAs CyclinD and p120 catenin in cells expressing eIF4G shRNA indicates a dependence on eIF4G abundance for the preferential synthesis of some IRES-containing mRNAs in equol-treated cells.

Effects on cell viability: Cell viability was determined using MTT assay in MDA-MB-435 cancer cells expressing control or eIF4G shRNA and treated with vehicle control or 25 μ M equol as described in the previous annual report. The ~1.4-fold increase in cell viability in response to 25 μ M equol remained constant in MDA-MB-435 cells expressing the eIF4G shRNA (Fig. 8).



Our results showed that the equol effect on cell viability is not eIF4G dependent. This result corroborates previous reports of eIF4G depletion causing only a small reduction in protein synthesis, cell viability, and only slightly impaired cell proliferation (21, 22). However, equol may exert additional pro-cancer effects on cell viability via increased c-Myc expression. The key transcription factor C-Myc regulates a large variety of cellular functions including cell cycle progression, protein synthesis, metabolism, apoptosis, and genomic instability (26, 27). c-Myc is altered in various human cancers, including breast cancer, and is regulated via several direct and indirect mechanisms (26-30). Our results show that equol upregulates mRNA, protein levels and the polysomal association of this important transcription factor, independent of eIF4G. These observations suggest that c-Myc may be a major contributor to the cancer promoting effects of equol. Therefore, this prompted us to examine the effect of knocking down c-Myc expression in MDA-MB-435 cells. This important finding and the data described in the present report result in a manuscript that is ready for submission to a peer reviewed journal (see appendix). Cells transfected with c-Myc siRNA demonstrated a 40% reduction in c-Myc protein expression compared to the cells with a control (Fig.9A). To investigate the role of c-Myc on cell viability, MDA-MB-435 cancer cells expressing control or c-Myc siRNA were treated with vehicle or 25µM equol and subjected to MTT assays. Figure 9 demonstrates that knockdown of c-Myc significantly decreased the viability of MDA-MB435 cells by 50% in vehicle treated cells and by 80% in equol treated cells. Therefore, the breast cancer promoting effects of equol may at least partially be due to c-Myc upregulation.



At present the mechanism by which equol upregulates c-Myc transcription is not known. Equol is structurally similar to estrogen with 80 times more ERβ affinity than their precursor, daidzein (31). C-Myc is a recognized estrogen and estrogen mimetics regulated oncogene. MDA-MB-435 has been shown to express splice variants of ER that may still be responsive to phytoestrogens (32). Therefore, equol may regulate c-Myc transcription via a variety of mechanisms that can involve estrogen receptor related pathways and crosstalk with growth factor receptor signaling. Future efforts should be directed toward the delineation of the molecular mechanisms by which equol regulates c-myc and eIF4G expression. These experiments may be extended to demonstrate that c-Myc is responsible for the increased eIF4G in response to equol. However, further experiments are needed to formulate such a conclusion.

KEY RESEARCH ACCOMPLISHMENTS:

1. eIF4G knockdown results in a marked reduction of the equol-mediated increase in protein synthesis initiation of specific IRES-dependent pro-cancer molecules: Cyclin D, Bcl-XI and p120 catenin.
2. The mRNA levels of CTNND1 (p120) and CCND1 (CyclinD) were not affected by equol treatment or eIF4G knockdown, indicating that cyclin D and p120 expression was regulated at the translational level.
3. Equol upregulates mRNA, protein levels and the polysomal association of c-Myc in cells with eIF4G knockdowns, showing that equol-mediated upregulation in c-Myc is independent of eIF4G.

4. The equol increased in IRES dependent translation in MDA-MB435 cells was significantly reduced when eIF4G was silenced.
5. The polysome profile in response to equol in MDA-MB-435 cells expressing eIF4G shRNA demonstrated that the total RNA associated with polysomes was decreased.
6. In cells expressing eIF4G shRNA, the reduction in eIF4G levels was reflected in decreased polysome association of CTNND1 and CCND1 in the light and heavy fractions.
7. The equol effect on cell viability is not eIF4G dependent.
8. The increase in cell viability in response to equol was not affected until c-Myc was also targeted by siRNA.
9. Our results implicate c-Myc and eIF4G in the cancer promoting effects of equol in metastatic breast cancer via regulation of protein synthesis initiation of molecules that control cancer progression.

REPORTABLE OUTCOMES:

The PI has completed the following milestones in her training program

Progress towards Ph.D. Degree

1. Wrote, edited, and successfully defended Ph.D. Dissertation – May 2014

Training

1. Supported partially by the DoD award, the PI spent a month at the laboratory of Dr. Robert Schneider, Ph.D. (Professor, New York University Langone Medical Center, NY), an expert in breast cancer and protein synthesis regulation, performing experiments related to the present project: polysome profiling of MDA-MB-435 cells expressing control or eIF4G shRNA following treatment with vehicle or equol.

Presentation of research at national and international conferences

1. The PI attended and presented a poster at the Experimental Biology Meeting (EB) in Boston, April 2013.

Abstracts

- Experimental Biology Meeting:

“Increased protein synthesis of IRES containing mRNAs, that induce cell survival and proliferation, by the daidzein metabolite equol may contribute to breast cancer malignancy.”

Authors: *Columba de la Parra* and Surangani F. Dharmawardhane Flanagan . Univ. of Puerto Rico Med. Science Campus, San Juan, PR.

Manuscript

Manuscript complete for submission to a peer reviewed journal

- **de la Parra, C.,** Schneider R.J and Dharmawardhane S. 2014. Equol, an isoflavone metabolite, increases cancer progression via upregulation of c-Myc and eIF4G. **See Appendix**

CONCLUSION:

Considerable work has been conducted on the role of dietary soy in cancer prevention, but not in the metastasis of established cancers; moreover the controversy of using soy foods in established cancers, or as substitutes for hormone replacement therapies continues. This research investigated the possible mechanism by which soy isoflavone affect established breast cancer. We have demonstrated during the first and second year of the award that in a highly metastatic cancer cell line MDA-MB-435, the effects of daidzein on increased mammary tumor growth, metastasis, and expression of eukaryotic protein synthesis initiation factor F (eIF4F) in a mouse model (14), is due to its metabolite equol (de la Parra, et al, 2012, J. Biol. Chem). We showed that equol, a metabolite produced by intestinal bacteria in some humans, is a pivotal soy isoflavone that contributes to cancer malignancy by increasing the expression of proteins that govern metastasis. We demonstrated that equol, may advance breast cancer progression via upregulation of the eukaryotic initiation factor 4G (eIF4G). The elevated eIF4G levels in response to equol were associated with an increase in cell viability and the translation of specific mRNAs with internal ribosome entry sites (IRES), including the transcription factor c-Myc, a central regulator of cancer malignancy. The data corresponding to the third year of funding period and summarized above demonstrated that siRNA targeting of eIF4G results in a marked reduction of the equol-mediated increase in protein synthesis initiation of specific IRES-dependent pro-cancer molecules, without affecting the increases in c-Myc levels and cell viability. However, knockdown of c-Myc abrogated the increased cell viability in response to equol. Overall, this study implicates c-Myc and eIF4G in the cancer promoting effects of equol in metastatic breast cancer via regulation of protein synthesis initiation of molecules that control cancer progression. Future efforts for better understanding the mechanism of action of soy isoflavones can be used for development of more effective prevention and therapeutic strategies for breast cancer. Hopefully, it can facilitate the public understanding of the impact of these products on breast cancer and ultimately impact the development of dietary guidelines for women at risk for breast cancer, breast cancer patients and survivors.

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APPENDICES

Equol, an isoflavone metabolite, increases cancer progression via upregulation of c-Myc and eIF4G

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Running Title: Equol upregulates cancer promoting molecules

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Key words: breast cancer, eukaryotic translation initiation, eukaryotic translation initiation factor 4G (eIF4G), c-Myc, cap-independent protein synthesis, equol, soy

Background: Equol, a daidzein metabolite, is a potent regulator of the cancer promoting effects of dietary daidzein.

Results: Knockdown of eIF4G or c-Myc demonstrated that the equol mediated c-Myc upregulation is central to the pro-cancer effects of equol.

Conclusion: Equol may promote cancer via c-Myc.

Significance: Consumption of daidzein may not be advisable for patients with ER (-) breast cancer.

ABSTRACT

Epidemiological studies implicate dietary soy isoflavones as breast cancer preventives, especially due to their anti-estrogenic properties. However, the role of soy isoflavones in established breast cancer has yet to be clarified. We previously reported that equol, a metabolite of the soy isoflavone daidzein, may advance breast cancer progression via upregulation of the eukaryotic initiation factor

4G (eIF4G). In estrogen receptor (-) metastatic breast cancer cells, elevated eIF4G levels in response to equol were associated with an increase in cell viability and the translation of specific mRNAs with internal ribosome entry sites (IRES), including the transcription factor c-Myc, a central regulator of cancer malignancy. Herein, we report that siRNA-mediated knockdown of eIF4GI results in a marked reduction of the equol-mediated increase in protein levels of specific IRES-dependent pro-cancer molecules, without affecting c-Myc levels or cell viability. However, knockdown of c-Myc abrogated the increased cell viability in response to equol, and resulted in a significant down regulation of eIF4GI, as well as decreases in the protein levels of some, but not all of the IRES-containing mRNAs that were increased by equol treatment. This study implicates c-Myc and eIF4G in the cancer promoting effects of equol in metastatic breast cancer via regulation of protein synthesis initiation of molecules that control cancer progression.

INTRODUCTION

Isoflavones are found in nutritionally relevant amounts in soybeans and comprise ~3.5 mg/g soy protein in traditional soy foods. Studies have reported 1-25 μM soy isoflavones in human circulation following consumption of soy products, which is sufficient for physiological activity (1-4). Due to the structural similarity to 17 β estradiol, these phytoestrogens have been extensively studied for their potential estrogenic or antiestrogenic effects in breast cancer (5, 6). Moreover, soy isoflavones may have additional estrogen-independent effects in aggressive estrogen receptor (ER) (-) breast cancers (5, 7).

Daidzein is the second most prominent isoflavone in soy and approximately 70% of daidzein can be metabolized by the intestinal

microflora to the metabolite equol. Only ~30-50% of humans have the gut microflora necessary to convert daidzein to equol; therefore, not all humans are affected by equol. Equol is also chemically similar to estrogen and has 80 times more ER β affinity than daidzein (8-10). However, the effects of equol, specifically in ER (-) breast cancers, or established aggressive breast cancers remains unclear (7, 11-13).

At high concentrations (50-350 μM), equol has been implicated with inhibition of cancer cell growth, invasion, cancer progression, and risk (14-18), while low physiological concentrations of equol increased cancer cell proliferation (13, 19). In ER (+) human breast cancer cells, equol increased estrogenic activity and cell proliferation, but did not affect tumor growth in mice (19-22). Dietary daidzein also failed to reduce mammary tumor growth in rats with ~1 μM equol in serum (23), while others have shown inhibitory effects of daidzein and equol on ER (+) breast cancer cells and tumors (24, 25). Thus, the data on breast cancer prevention or promotion by daidzein and equol is controversial and appears to be concentration and ER status dependent. Accordingly, an understanding of the molecular mechanisms of equol that regulate breast cancer should aid in recommendations on soy consumption for breast cancer patients.

To this end, we tested the effect of individual and combined soy isoflavones on nude mice with mammary tumors established from ER (-) cancer cells, and reported that dietary daidzein increased mammary tumor growth and metastasis (26). Our recent in vitro studies demonstrated that equol is the active metabolite of daidzein that increased breast cancer malignancy, primarily via upregulation of the eukaryotic initiation factor eIF4G and related translation of pro-cancer molecules (27).

Translation control has received increased attention during recent years due to its emerging

significance in cancer development and progression (28). Since translation initiation is the rate limiting step, regulation of translation primarily occurs at this stage. Accordingly, levels of eukaryotic initiation factors, especially members of the eIF4F complex eIF4E and eIF4G, are frequently elevated in human cancers, and have been associated with poor prognosis and outcome (28-30). Overexpression of eIF4G1 is critical for cap-independent translation initiation in eukaryotic cells that bypasses the requirement for cap-dependent scanning and allows the 40S ribosome to be directly recruited via Internal Ribosome Entry Sites (IRESs). It is thought that under the physiological stress conditions that exist in large tumors (i.e. growth arrest, amino acid starvation, mitosis, hypoxia), cancer cells rely on cap-independent and IRES-dependent translation of a subset of pro-growth, proangiogenic and pro-survival molecules such as, eIF4G, BCL2, Bcl -X1, C-Myc, p120 catenin, vascular endothelial growth factor (VEGF) etc. These proteins have mRNAs that contain IRES elements, and thus may be translated via cap-dependent or -independent mechanisms (28, 31-33).

We recently demonstrated that equol increased eIF4G levels in MDA-MB-435 ER (-) metastatic breast cancer cells, and that this upregulation was associated with an increased translation of IRES-containing mRNAs that induce cell survival and cell proliferation, and thus promote cancer malignancy. Noteworthy was the increase of the transcription factor c-Myc (27). c-Myc is overexpressed in a variety of human cancers and plays an important role in multiple signaling pathways including cell growth, cell proliferation, metabolism, ribosome biogenesis, microRNA regulation, cell death, and cell survival (34-36).

The objective of the present study was to determine the relative contribution of equol-mediated eIF4G and c-Myc upregulation to the cancer promoting effects of equol. To clarify whether equol mediated eIF4G upregulation leads

to preferential synthesis of c-Myc, which has an IRES site (37), or if c-Myc upregulation by equol leads to eIF4G transcription, as shown in (38), we investigated the effects of silencing eIF4G or c-Myc in equol-treated metastatic breast cancer cells. Herein, we show that targeting eIF4G1, results in a marked reduction in IRES dependent translation, polysomal association of mRNAs, and protein levels of specific IRES containing mRNAs of pro-cancer molecules. The increase in cell viability in response to equol was not affected until c-Myc was also targeted by siRNA, which in addition, significantly downregulated eIF4G levels. Taken together, this data implicate c-Myc, and the consequent increase in eIF4G, followed by protein synthesis of pro-cancer molecules, in the effects of equol on breast cancer progression.

EXPERIMENTAL PROCEDURES

Cell culture – Metastatic variant of MDA-MB-435 (ER-) (gift of Dr. Danny Welch, The University of Kansas Cancer Center) and Hs578t (ER-) metastatic human breast cancer cells (American Type Culture Collection, Manassas, VA) were maintained in complete culture medium: Dulbecco's Modified Eagle Medium (Invitrogen, Houston, TX) supplemented with 10% fetal bovine serum (Invitrogen, Houston, TX) at 37 °C in 5% CO₂.

Cell treatment – Quiescent metastatic cancer cells were treated with 0 (vehicle, 0.1% DMSO) or 25 µM of (R,S) Equol (LC Laboratories, Woburn, MA) in DMEM and 5% FBS media for 24 h.

Western blotting – Cells were lysed and western blotted, as described in (26). Primary antibodies to eIF4E, eIF4G1, c-Myc, p120 catenin, Bcl-X1, Cyclin D, GAPDH, JunB and β actin (Epitomics, Burlingame, CA, Cell Signaling, Danvers, MA, Sigma-Aldrich Comp., St Louis, MO) were used. The integrated density of positive bands was quantified using Image J software, as described in (26).

Cell viability assay – Cell viability was determined by the CellTiter 96 Non-Radioactive Cell Proliferation kit according to manufacturer's instructions (Promega, Madison, WI). Briefly, quiescent 1×10^5 MDA-MB-435 cells were added to the wells of a 96-well plate and treated for 24 h with vehicle or 25 μ M equol. Following equilibration, 15 μ L/well of MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent was added and the plates incubated at 37°C for 4 hours. Stop solution (100 μ L) was added to each well, and the plates were incubated to facilitate solubilization of newly formed formazan salts. The absorbance at 570 nm was measured using an ELISA plate reader.

Real-Time reverse transcriptase polymerase reaction (RT-PCR) analysis – Real-time quantitative RT-PCR analysis was performed as described in (27). Briefly, total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Valencia, CA). RNA concentration was detected using a NanoDrop (NanoDrop Technologies, Wilmington, DE). RNA (0.5 μ g) was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR primers were as follows. MYC: forward, 5'-TTCTCAGAGGCTTGCGGGAAA-3', reverse, 5' TGCCTCTCGCTGGAATTACTACA-3'. Beta 2microglobulin (B2M): forward, 5'-GGCTATCCAGCGTACTCCAAA-3', reverse, 5'-CGGCAGGCATACTCATCTTTT-3'. GAPDH: forward, 5'-TTGCCATCAATGACCCCTTCA 3', reverse, 5'CGCCCCACTTGATTTTGGA-3'. CCND1: forward, 5'-TGGTGAACAAGCTCAAGTGGA-3'. reverse, 5' TGATCTGTTTGTCTCCTCCGCCT-3'. EIF4G: forward, 5'-TTGTGGATGATGGTGGCT-3' reverse, 5'-TTATCTGTGCTTTCTGTGGGT-3'. CTNND1: forward, 5'-TCCAGCAAACGATACAGTGG-3', reverse, 5'-GAACCACCTCTGGCTGAAAT-3'. Real-time reactions were performed using iQ SYBR-Green

PCR Master Mix (Bio-Rad, Hercules, CA). The amplification reaction was performed for 40 cycles (10s at 95 °C, 30s at 59°C, and 30s at 72 °C). B2M mRNA was used as an internal control. The fold change was determined by the $2^{-\Delta\Delta CT}$ method as described in (26, 27).

Polysomal fractionation– shRNA transfected MDA-MB-435 cells (control or eIFGI), treated with vehicle or equol, were used for polysome profiling, as described in (32). Cells were pre-treated with 100 μ g/ml cycloheximide (Calbiochem), washed twice in PBS with 100 μ g/ml cycloheximide, pelleted, and resuspended in 700 μ L polysome isolation buffer (200 mM Tris, pH 7.5, 100 mM NaCl, and 30 mM MgCl₂). After 5 min incubation, 250 μ L detergent buffer (1.2% Triton, 0.2 M sucrose in polysome isolation buffer) was added and cells were lysed. The clarified lysates were layered onto 10–50% sucrose (Sigma-Aldrich) gradients and sedimented at 36,000 rpm for 2 h in a SW40 rotor (Beckman Coulter) at 4°C. Gradients were collected as 15 \times 750 μ L fractions by pumping 60% sucrose into the bottom of the gradient and collecting from the top using an ISCO fraction collector while simultaneously monitoring absorbance at 254 nm. RNA was isolated by extraction with phenol/chloroform. Fractions 4–12, representing polysomes, were pooled and classified as light polysome fractions (4-7) and heavy polysome fractions (8-12). RNA preparations from each fraction were subjected to qRT-PCR for C-MYC, CTNND1 (p120-catenin), CCND1 (cyclin D), EIF4G, GAPDH and B2M, as described above.

shRNA/siRNA transfection– eIF4G knockdown using shRNA adenovirus vectors were conducted as described in (33). MDA-MB-435 and Hs578t cells were infected two times over four days with nonsilencing (control) or eIF4GI-silencing Adenovirus (Ad) shRNA vectors at a multiplicity of infection (MOI) of 100, then treated with vehicle or 25 μ M equol for 24 h. Control or c-Myc siRNA (Santa Cruz, CA) were transfected in to

MDA-MB-435 and Hs578t cells using lipofectamine (Invitrogen) according to the manufacturer's instructions, followed by treatment with vehicle or equol for 24h.

IRES-dependent protein synthesis– MDA-MB-435 cells with control or eIF4G knockdowns were transfected with a bicistronic reporter system or control plasmid containing the luciferase constructs without an IRES, using Lipofectamine 2000 (Invitrogen), as per manufacturer's directions. As described in (33), this plasmid contains a cap-dependent Renilla luciferase (RLuc) followed by a 5'UTR containing the p120 catenin IRES driving a firefly luciferase (FLuc). 24 h following transfection, cells were treated with equol for an additional 24 h. The relative IRES activity was analyzed as 570 nm FLuc/480 nm RLuc in a luminometer using a dual luciferase assay kit (Promega, Corp., Madison, WI), according to manufacturer's instructions.

Statistical analysis– Data was analyzed and reported as mean \pm SEM of at least three independent experiments. Statistical analyses were done using Microsoft Excel. Differences between means were determined using Student's t-Test and values <0.05 were considered significant.

RESULTS

eIF4G knockdown partially rescues the pro-cancer effects of equol. – We recently reported that the daidzein metabolite equol, through the up-regulation of eukaryotic protein synthesis initiation factor eIF4G, may specifically direct the synthesis of IRES containing mRNAs that induce cell survival and proliferation, and promote cancer progression (27). To investigate whether equol regulates breast cancer cell malignancy via eIF4G, we determined the effects of equol on MDA-MB-435 and Hs578t cells with eIF4G knockdowns. Using eIF4G shRNA vectors, we were able to knockdown eIF4G protein expression by $\sim 40\%$ in Hs578t and 65% in MDA-MB-435 cells,

compared to cells expressing vector controls, in both vehicle and equol-treated cells. The ~ 1.4 - 1.7 -fold increase in eIF4G levels in response to equol was effectively abolished by the eIF4G siRNA targeting, indicating that this strategy successfully inhibited all de novo synthesis of eIF4G (Fig. 1A). However, the ~ 1.4 - 1.7 fold increase in cell viability in response to $25\mu\text{M}$ equol remained constant in MDA-MB-435 and Hs578t cells respectively expressing the eIF4G shRNA (Fig. 1B, C). These results indicate that the equol effect on cell viability is not eIF4G dependent.

Since equol upregulates protein expression of IRES containing pro-cancer molecules (27), cells expressing control or eIF4G knockdown were analyzed for protein expression levels of members of the eIF4F complex and pro-cancer molecules from IRES containing mRNAs (see Fig. 2A-C). As expected, $25\mu\text{M}$ equol increased eIF4G (~ 1.4 , 1.6 -fold), c-Myc (~ 1.5 , 1.8 -fold), Cyclin D (~ 1.4 , 1.5 -fold), Bcl-XL (~ 1.3 , 1.5 -fold), and p120 catenin (~ 1.3 , 1.6 fold) in a statistically significant manner, respectively, in the Hs578t cells and MDA-MB-435 cells expressing control shRNA. The cells expressing eIF4G shRNA demonstrated a ~ 40 - 50% reduction in eIF4G in vehicle treatments and a 80 - 120% reduction in equol-treated cells, while the eIF4E levels remained unchanged. Knockdown of eIF4G also caused a marked $\sim 40\%$ - 60% reduction in protein expression levels of mRNAs with IRESs: Cyclin D, Bcl-XL and p120 catenin, from both vehicle and equol treated cells; while the protein expression of GAPDH, JunB and Actin from mRNAs with short 5'UTR lacking an IRES were not affected by equol treatment or eIF4G silencing. These results suggest that elevated eIF4G levels are essential for the significant increase in protein expression of IRES-containing cancer promoting molecules, in response to equol treatment. However, the equol-mediated ~ 1.5 - 1.7 -fold increase in c-Myc, an

IRES containing mRNA, remained elevated regardless of the eIF4G knockdown (Fig. 2).

To determine whether silencing eIF4GI may affect the molecules tested in Fig. 2 A,B at the transcriptional level, we quantified the mRNA levels of eIF4G, C-MYC, CTNND1 (p120) and CCND1(CyclinD) from MDA-MB-435 cancer cells expressing control or eIF4G shRNA, treated with vehicle or 25 μ M equol. Figure 2C demonstrates that as previously reported by us from MDA-MB-435 mammary tumors and cells (26, 27), equol increased eIF4G transcription by ~1.8-fold. This increase was abolished in cells expressing eIF4G shRNA, where eIF4G expression was reduced by ~3-fold in both vehicle and equol-treated cells. The mRNA levels of CTNND1 (p120) and CCND1 (CyclinD) were not affected by equol treatment or eIF4G knockdown, indicating that cyclin D and p120 expression was regulated at the translational level. However, mRNA levels of c-Myc increased (~1.9-fold) in response to equol in both control and cells with eIF4G knockdowns, confirming that the equol-mediated upregulation in c-Myc protein levels is independent of eIF4G (Fig 2).

In order to determine whether the elevated eIF4G in response to equol might be necessary for an efficient translation of IRES elements, we performed dual luciferase assays for cap-dependent and IRES-dependent protein synthesis on MDA-MB-435 cells expressing control or eIF4G shRNA, treated with vehicle or equol. The data shown in Figure 3, demonstrates that equol increased IRES dependent translation in cells expressing control shRNA by ~1.7-fold, while this increase was significantly reduced when eIF4GI was silenced. Vehicle treatment of cells with eIF4GI knockdown also resulted in a slight, but not statistically different, decrease in IRES activity (data not shown). Thus, the elevated protein synthesis of mRNAs with IRESs can be attributed to the increased eIF4G in response to equol.

To investigate whether protein synthesis initiation might be sensitive to the reduction in eIF4GI levels, we performed polysome profiles for control and eIF4GI-silenced MDA-MB-435 cells treated with vehicle or equol. As previously reported (27), figure 4A shows that equol increased the total mRNA associated with the polysomal fractions compared with vehicle, especially in the cells expressing a control shRNA. The total RNA associated with polysomes was slightly decreased in equol-treated MDA-MB-435 cells with eIF4GI knockdown compared to cells expressing control shRNA. In the vehicle treated cells, eIF4G knockdown resulted in decreased association of RNA with both light and heavy polysomal fractions, compared to cells expressing a control shRNA.

To identify specific mRNAs that were differentially associated with the polysome fractions in control or eIF4G knockdown cells, real-time quantitative (q) RT-PCR was performed for the IRES containing mRNAs: CTNND1, CCND1, EIF4G, C-MYC, and the non IRES containing housekeeping genes GAPDH and B2M. Figure 4B demonstrates that in the MDA-MB-435 cells expressing control shRNA, equol treatment increased the association of eIF4G with both light and heavy polysomal fractions (~1.7-fold), while the association of c-Myc, CTNND1, and CCND1 with the heavy polysomal fraction was increased by ~1.5-1.8-fold. In MDA-MB-435 cells expressing eIF4G shRNA, a ~2.5-fold reduction in eIF4GI levels was reflected in decreased polysome association of CTNND1 and CCND1 by ~ -1.2 fold in the light fractions and by ~ - 1.4 fold in the heavy fractions. However, the equol-stimulated c-Myc mRNA levels remained increased by 1.4 and 1.7 fold in the light and heavy fractions compared with vehicle controls, even in the cells with eIF4G knockdown. The IRES-negative GAPDH mRNA was also not affected by eIF4GI knockdown or equol treatment. The observed down-regulation of equol-mediated

CyclinD and p120 catenin protein expression (Fig. 2A) and decreased polysome association of their mRNA in the cells with eIF4G knockdown (Fig. 4B), indicates a dependence on eIF4G abundance for the preferential synthesis of some IRES-containing mRNAs in equol-treated cells.

c-Myc knockdown reduces eIF4G levels and abrogates the effects of equol on cell viability. Our results show that equol upregulates mRNA and protein levels and the polysomal association of the key transcription factor c-Myc, independent of eIF4G (Figs.2,4). These results were not affected by the knockdown of eIF4G, indicating a central role for c-Myc as a major contributor to the cancer promoting effects of equol. Therefore, we tested the effect of knocking down c-Myc expression in Hs578t and MDA-MB-435 cells. Cells transfected with c-Myc siRNA demonstrated a 30-40% reduction in c-Myc protein expression compared to the cells with a control siRNA when treated with vehicle. Equol treatment, which resulted in a 1.5-fold increase in c-Myc expression in both cell lines expressing a control siRNA, was completely abolished in the cells expressing c-Myc siRNA, with 60% of the c-Myc protein remaining intact. This result indicates that the siRNA targeting of c-Myc is sufficient to inhibit the de novo protein synthesis of c-Myc in response to equol, without affecting the basal levels, i.e. the 60% of c-Myc that is insensitive to siRNA targeting (Fig. 5A). As shown in Fig. 5A, protein expression of eIF4G and cyclin D resulted in a parallel significant downregulation in the cells with c-Myc knockdown, indicating direct regulation of eIF4G mRNA expression by c-Myc. Interestingly, the protein levels of Bcl-Xl and p120 catenin were not affected by the c-Myc knockdown and demonstrated statistically significant increases in response to equol in cells with control or c-Myc siRNA in MDA-MB-435 cells. In the Hs578t cell line, only p120 catenin expression was not affected by the c-Myc knockdown. In this cell line,

protein expression of eIF4G, cyclin D, and Bcl-Xl were downregulated following c-Myc knockdown.

A number of genes involved in cell growth, apoptosis and metabolism have been implicated as targets of c-Myc regulation (39-41). To investigate the role of c-Myc on cell viability, Hs578t and MDA-MB-435 cancer cells expressing control or c-Myc siRNA were treated with vehicle or 25 μ M equol and subjected to MTT assays. Figure 6 demonstrates that knockdown of c-Myc significantly decreased the viability of MDA-MB-435 cells by 50% in vehicle treated cells and by 50-80% in equol treated cells. Therefore, the breast cancer promoting effects of equol may at least partially be due to c-Myc upregulation.

DISCUSSION

Dysregulation of translational control can have oncogenic consequences by altering global control of protein synthesis as well as selective translation of a subset of mRNAs important for cell growth, survival, metastasis and proliferation (28, 42). Accordingly, eIF4F complex members eIF4E and eIF4G have been shown to be overexpressed in advanced cancer. Upregulation of eIF4G in aggressive breast cancers may preferentially enhance cap- independent IRES- mediated translation, especially, when the cap-dependent scanning mechanism of translation initiation is compromised by stress conditions (43-45).

Therefore, our finding that eIF4G is upregulated by the dietary soy isoflavone equol in MDA-MB-435 mammary tumors in nude mice as well as metastatic breast cancer cells in vitro, implicated the equol-stimulated eIF4G in translational control (26, 27). We also showed that equol treatment of breast cancer cells resulted in increased IRES-dependent protein synthesis, and increased protein expression and polysome association of a number of mRNAs with IRESs coding for proteins that regulate cancer cell survival, proliferation, and invasion (27). In this

study, we tested the hypothesis that elevated eIF4G in response to equol directly contributes to the increased survival properties of breast cancer cells.

However, the increased viability of metastatic cancer cells in response to equol was insensitive to eIF4GI knockdown. This result corroborates previous reports of eIF4G depletion causing only a small reduction in protein synthesis, cell viability, and only slightly impaired cell proliferation (32, 33). Nevertheless, eIF4G knockdown has been shown to decrease the expression of the IRES containing mRNA p120 catenin that has been implicated in invasion, as well as the resulting invasion of inflammatory breast cancer cells (33).

Confirming this report, we found eIF4G knockdown in metastatic breast cancer cells to result in a significant reduction in the equol-mediated increase in protein expression of p120 catenin, Cyclin D, and Bcl-Xl without affecting their mRNA levels. These molecules contain IRESs in their mRNA and have been shown to be under eIF4G regulation during cancer progression (33, 46-48). The decreased IRES-dependent protein synthesis and the reduced polysomal association of Cyclin D and p120 mRNA also validates the hypothesis that elevation of eIF4GI by equol treatment favors the translation of these IRES-containing cancer promoting molecules. When eIF4G was knocked down by 50-60%, we observed a resultant 50-60% decrease in the synthesis of IRES-containing mRNAs. The eIF4G knockdown was sufficient to reduce 100% of the equol-mediated increases in Cyclin D, Bcl-Xl, and p120 catenin, indicating that the expression of these IRES containing mRNAs, but not c-Myc, were specifically dependent on elevated eIF4G levels.

A central finding from our research is that equol increases gene and protein levels of eIF4GI and C-Myc. Intriguingly, the c-Myc and eIF4E protein levels were not affected by the silencing of

eIF4GI. The key transcription factor C-Myc regulates a large variety of cellular functions including cell cycle progression, protein synthesis, metabolism, apoptosis, and genomic instability through several signaling pathways (35, 49). c-Myc is altered in various human cancers, including breast cancer, and is regulated via several direct and indirect mechanisms (35, 49-52). We found that decreasing levels of eIF4GI was not sufficient to affect c-Myc levels, nor abolish the increase in cell viability in response to equol. Therefore, equol may activate c-Myc transcription independent of eIF4G upregulation.

Overall, c-Myc appears to be a major player in the cancer promoting effects of equol in the MDA-MB-435 and Hs578t metastatic cancer cells. Although both eIF4E and eIF4G are transcriptionally regulated by c-Myc (38, 53), we found a significant reduction in eIF4G and a trend in eIF4E reduction in the cells with c-Myc siRNA. c-Myc knockdown resulted in a significant decrease in Cyclin D levels, which were also reduced in cells with eIF4G knockdown. Validating the role of c-Myc in control of cell viability in several cancers (54, 55), knockdown of c-Myc to basal levels of expression in the Hs578t and MDA-MB-435 cells, completely abolished the increased cell viability in response to equol. Moreover, c-Myc knockdown resulted in the abrogation of equol-mediated eIF4G and Cyclin D upregulation, but not Bcl-Xl and p120 catenin in MDA-MB-435 cells. Since elevated Bcl-Xl and p120-catenin levels in response to equol were inhibited by direct eIF4G knockdown, as well as with c-Myc knockdown, this result signifies that equol mediated c-Myc upregulation leads to the transcriptional activation of eIF4G. The resultant elevated eIF4G levels are predicted to induce preferential cap-independent synthesis of IRES-containing mRNAs such as Cyclin D, Bcl-Xl, and p120 catenin. However, when c-myc is knocked down and the elevated eIF4G levels in response to equol are completely abolished, cap-dependent

protein synthesis may account for the increased Bcl-Xl and p120 expression.

At present the mechanism by which equol upregulates c-Myc transcription is not known. Equol is structurally similar to estrogen with 80 times more ER β affinity than their precursor, daidzein (56). C-Myc is a recognized estrogen and estrogen mimetics regulated oncogene. However, unlike other estrogen-dependent genes, the mechanism by which c-myc is regulated is not completely understood (51). C-Myc transcription is regulated by a number of cell surface receptors that include wnt/ β -catenin and growth factor or signaling that activates transcription factors such as TCF/LEF and AP-1, known to regulate c-Myc expression in breast cancer (34, 51, 57). Even though the role of equol in wnt signaling is not yet established, equol has been implicated in enhanced AP-1 activity (58). In our study, we used ER-negative breast cancer cell lines, however these cell lines still expresses steroid receptors and may even express estrogen related receptors (56, 59). Moreover, MDA-MB-435 has been shown to express splice variants of ER that may still be responsive to phytoestrogens (60). Therefore, equol may regulate c-Myc transcription via a variety of mechanisms that can involve estrogen receptor related pathways and crosstalk with growth factor receptor signaling.

c-Myc regulates approximately 15% of the genome, and can also regulate multiple stages of ribosome biogenesis including the expression of translation initiation factors that are important for both cap-dependent and cap-independent translation (39, 61). C-Myc is a known transcriptional regulator of Cyclin D, which

explains our results with decreased Cyclin D expression and cell viability in breast cancer cells expressing c-Myc siRNA. Taken together, this study demonstrates that equol may increase cancer progression via upregulation of c-Myc transcription leading to c-Myc dependent and -independent eIF4G-mediated protein synthesis initiation of pro-cancer molecules and increased cancer cell survival.

In conclusion, we have shown that equol may exert additional pro-cancer effects via increased c-Myc expression. eIF4G knockdown resulted in a significant reduction in protein expression levels of IRES containing molecules as well of the IRES dependent translation capacity without affecting cell viability or overall translation. Knockdown of c-Myc abrogated the increased cell viability in response to equol and resulted in the abolition of equol-mediated eIF4G and Cyclin D upregulation. Therefore, this study implicates equol increases in metastatic cancer progression via upregulation of c-Myc and the consequent increase in eIF4G followed by protein synthesis of pro-cancer molecules. This study contributes to the knowledge on the possible mechanisms by which soy isoflavones may affect breast cancer by demonstrating a key role for c-Myc in the pro-cancer effects of equol. However further studies are needed to determine the exactly mechanism by which equol upregulates c-Myc transcription.

Acknowledgements—We wish to thank Dr. Deborah Silvera for providing the eIF4G shRNA constructs.

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FOOTNOTES

* This research was supported by grant numbers US Army/BCRP W81XWH-11-1-0199 to CD and NIH/NIGMS SC3GM084824 to SD.

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²The abbreviations used are: eIF, eukaryotic protein synthesis initiation factors; IRESs, internal ribosome entry sites; 4E-BP, 4E binding protein; ER, estrogen receptor; O-DMA, O-desmethyldangolensin; RT-PCR, Real-Time reverse transcriptase polymerase reaction; CCND1, Cyclin D1; CTNNB1, Catenin (cadherin-associated protein), beta 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JUN, Jun oncogene; VEGF, vascular endothelial growth factor, RLuc, *Renilla* luciferase, FLuc, firefly luciferase; shRNA, short hairpin RNA; siRNA, small interfering RNA; UTR, untranslated region.

FIGURE LEGENDS

FIGURE 1. Cell viability in response to equol in MDA-MB-435 and Hs578t cells expressing control or eIF4G shRNA. Quiescent MDA-MB-435 and Hs578t cells were infected with non-silencing control or eIF4G-silencing (eIF4G) Adenovirus shRNA vectors, then treated with vehicle or 25 μ M equol for 24 h. Cells were lysed and western blotted for eIF4G protein or subjected to a MTT assay. (A) Representative western blot for MDA-MB-435 cells. B. MDA-MB-435 cell viability as a percentage of vehicle treatments (100%). C. Hs578t cell viability as a percentage of vehicle treatments (100%). Values show mean \pm SEM (N = 3). An asterisk indicates statistical significance at $p \leq 0.05$.

FIGURE 2. Protein and gene expression of selected molecules in response to equol in MDA-MB435 and Hs578t cells expressing control or eIF4G shRNA. MDA-MB-435 and Hs578t cells were infected with nonsilencing (control) or eIF4G silencing (eIF4G) Adenovirus (Ad) shRNA vectors, then treated with vehicle or 25 μ M equol for 24 h. Lysates were western blotted for the indicated proteins or gene expression and quantified by qRT-PCR. (A) Representative western blots. (B) Fold changes of protein expression compared to vehicle as calculated from the integrated density of positive bands normalized for actin expression, in MDA-MB-435 cells. (C) Fold changes in EIF4G, MYC, CTNND and CCND1 mRNA. B2M was used as an internal control. Results are shown as fold-changes in equol-treated MDA-MB-435 cells relative to vehicle controls (N=3). An asterisk indicates statistical significance at $p \leq 0.05$.

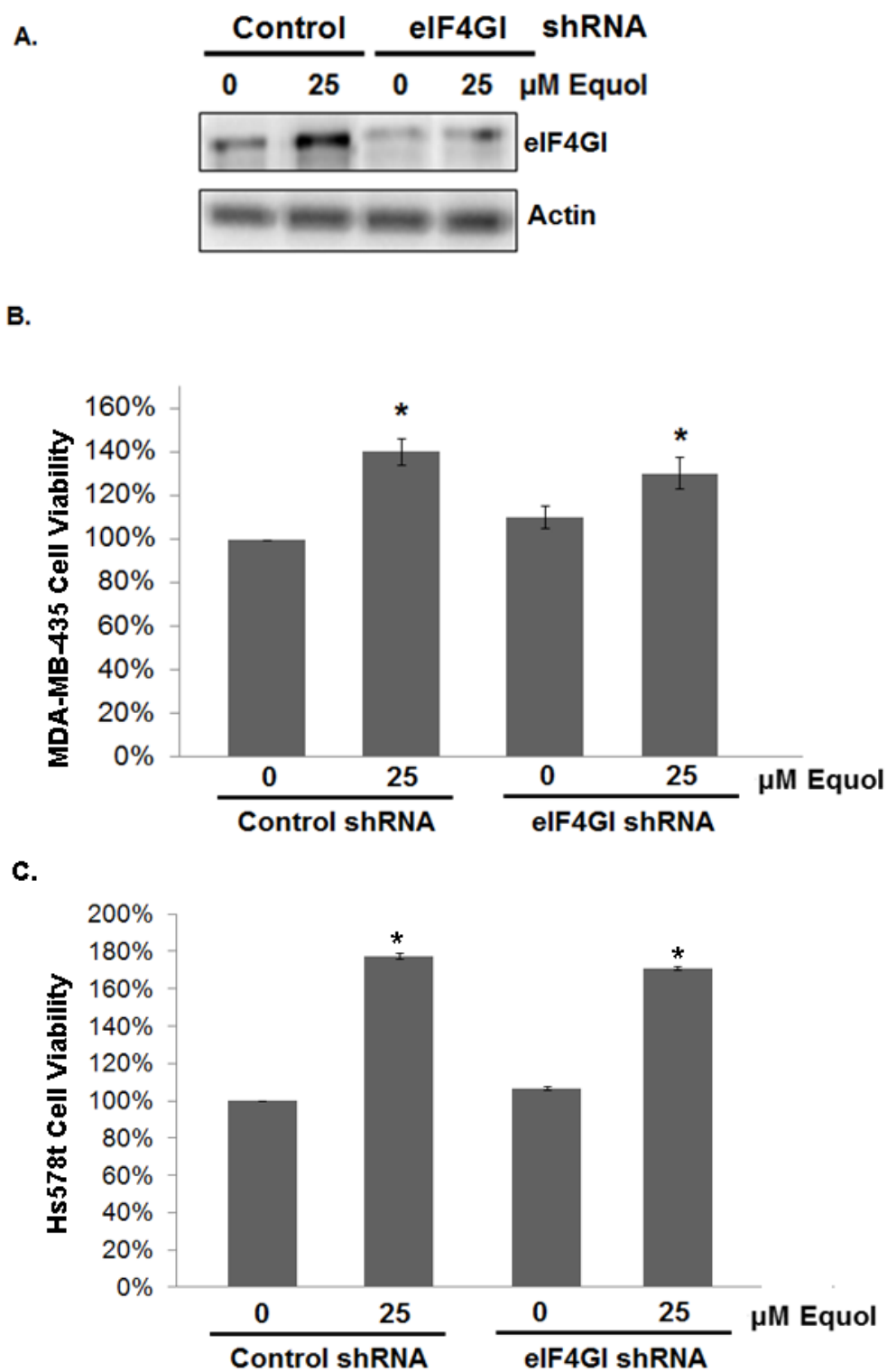
FIGURE 3. Effect of eIF4G knockdown on IRES-dependent protein synthesis in response to equol. MDA-MB-435 cells with control or eIF4G knockdowns expressing a plasmid with a cap-dependent *Renilla* luciferase (RLuc) followed by a 5'UTR containing the p120 catenin IRES driving a firefly luciferase (FLuc) or control plasmid without an IRES, were treated with vehicle or equol for 24 h. Cells were lysed, and the relative IRES activity analyzed as 570 nm FLuc/480 nm RLuc. IRES activity was quantified relative to control activity for vehicle or equol treated cells. Results show fold change in IRES activity compared to vehicle for N=3 \pm SEM. An asterisk indicates statistical significance of $p \leq 0.05$.

FIGURE 4. Analysis of polysome profiles in response to vehicle or equol in MDA-MB-435 cells expressing control or eIF4G shRNA. Total cell lysates from vehicle control or 25 μ M equol-treated MDA-MB-435 cells expressing control or eIF4G shRNA were loaded onto 10-50% sucrose gradients and light (4-7) and heavy polysome fractions (8-12) collected. (A) Polysome profiles. (B) C-MYC, EIF4G, CNND1, CTNND and GAPDH mRNA associated with the polysome fractions, as detected by qRT-PCR. B2M was used as an internal control. Results are shown as fold-changes in equol-treated cells relative to vehicle controls (N=3). An asterisk indicates statistical significance of $p \leq 0.05$.

FIGURE 5. Expression of pro-cancer molecules following equol in MDA-MB 435 and Hs578t with c-Myc knockdown. Quiescent MDA-MB-435 and Hs578t cells expressing control or c-Myc siRNA were treated with vehicle or equol for 24h. Lysates were western blotted for the indicated proteins. (A) Representative western blots. (B) Fold changes of protein expression compared to vehicle as calculated from the integrated density of positive bands normalized for actin expression (N=3). An asterisk indicates statistical significance of $p \leq 0.05$.

FIGURE 6. Effect of c-Myc knockdown and equol on cell viability. 48 h after control or c-Myc siRNA transfection, (A) MDA-MB-435 or (B) Hs578t cells were treated with vehicle or 25 μ M equol for 24 h. Cells were lysed and subjected to a MTT assay. Results are shown relative to vehicle (100%). N=3 for all experiments. A single asterisk indicates statistical significance of $p \leq 0.05$. Two asterisks indicate statistical significance of $p \leq 0.01$.

Figure 1.



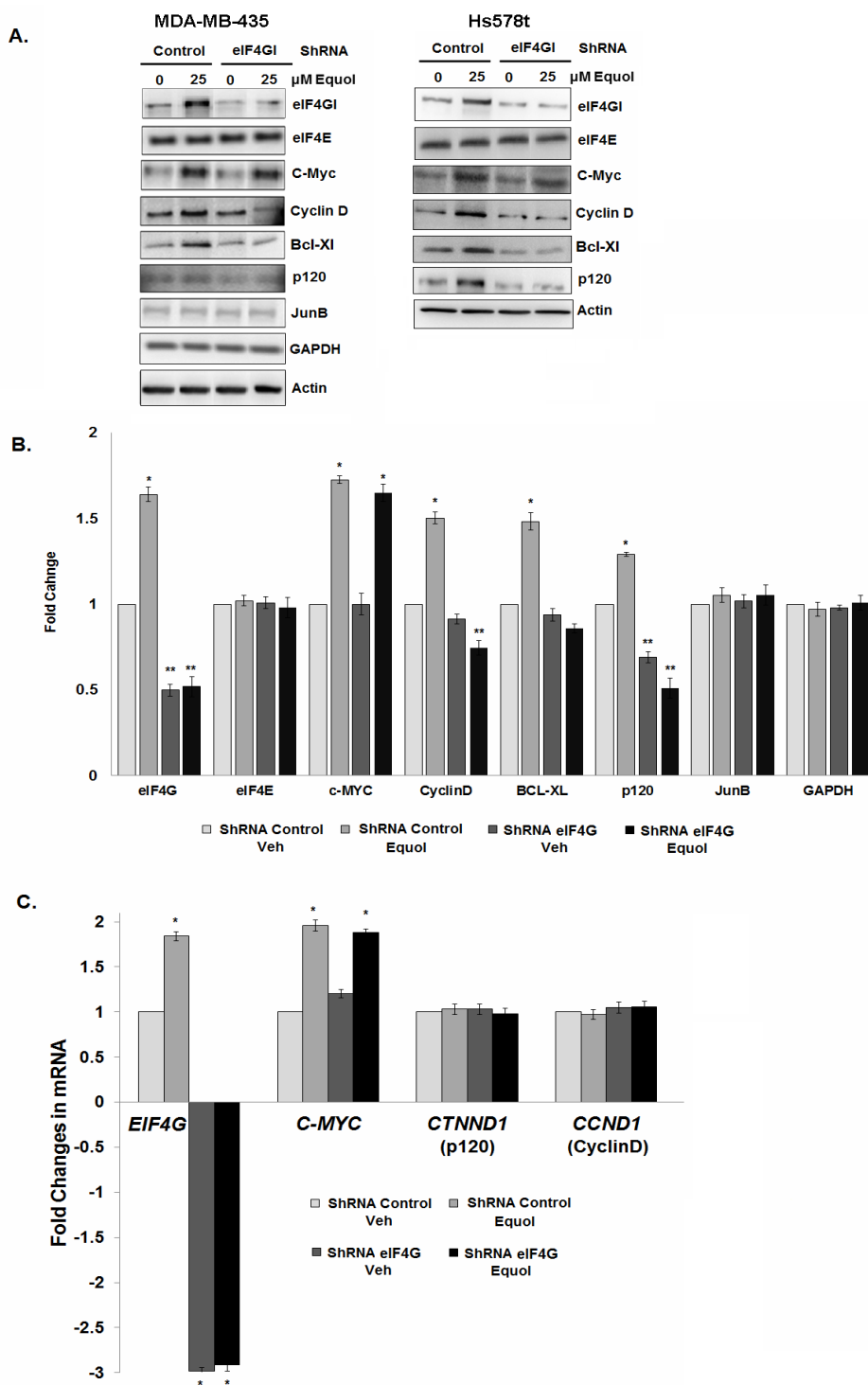


Figure 3.

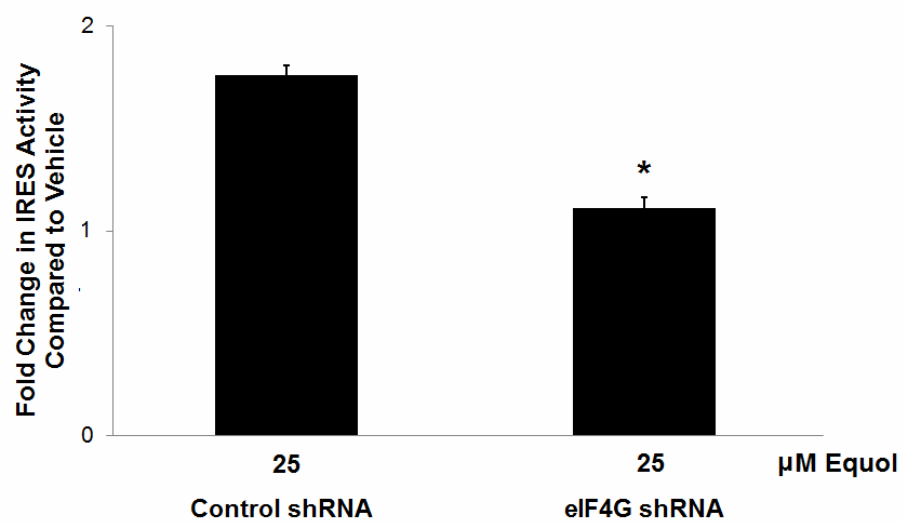


Figure 4.

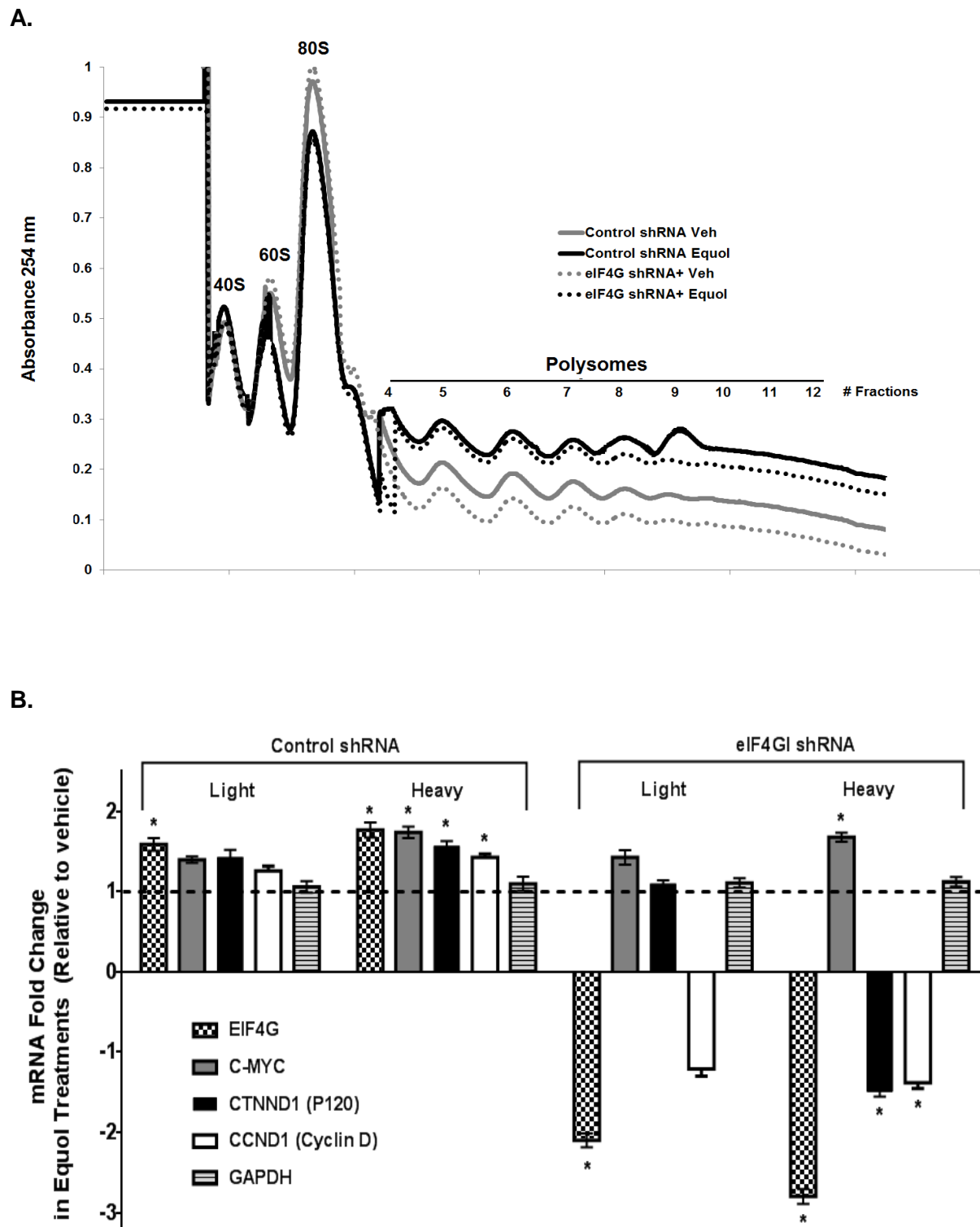


Figure 5.

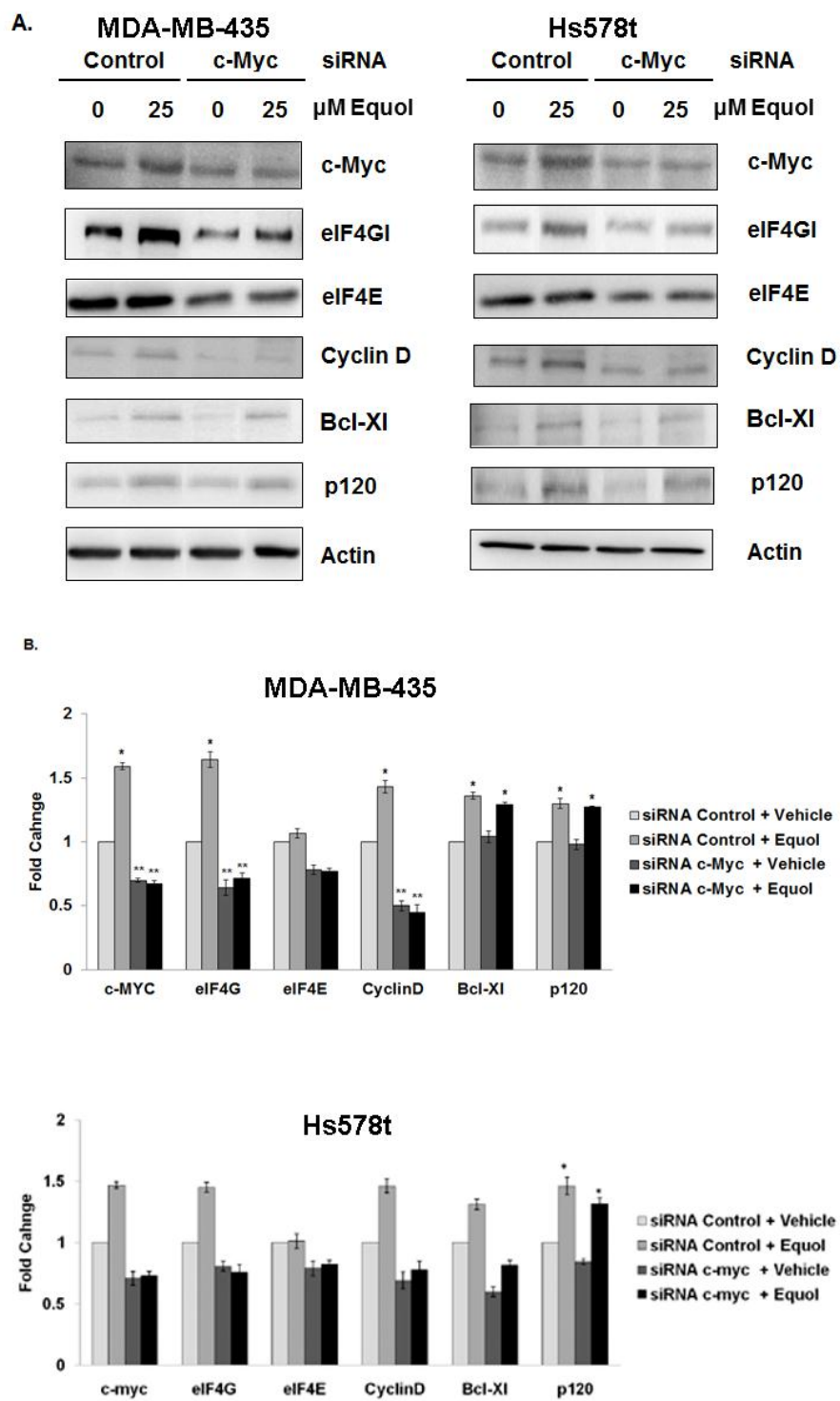


Figure 6.

